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# A VASODILATOR INNERVATION TO THE CENTRAL ARTERY OF THE RABBIT EAR

S. KALSNER
Department of Pharmacology, Faculty of Medicine, University of Ottawa, Ottawa, Ontario, Canada

1 The possibility of a vasodilator innervation to the isolated and perfused central artery of the rabbit ear was examined.

2 Stimulation of the periarterial nerves in the presence of noradrenaline or other agonist used to maintain a partial constriction of the ear artery, led to a decrease in intraluminal flow followed after the cessation of stimulation by an increase in flow beyond the pre-stimulation level.

3 After blockade of adrenergic transmission with bretylium or guanethidine or of the $\alpha$- and $\beta$-adrenoceptors with phentolamine and propranolol, stimulation of the periarterial nerves in the presence of a background tone, led to a clearly detectable vasodilatation. This dilatation was not blocked by treatment with atropine or mepyramine; nor was it enhanced by physostigmine.

4 Pretreatment of rabbits with reserpine (2 mg/kg) to deplete catecholamine stores, eliminated both the vasoconstrictor and vasodilator responses to nerve stimulation. However, a lower dose of reserpine (0.2 to 0.5 mg/kg) selectively eliminated the vasoconstrictor component of periarterial nerve activation.

5 The ear artery dilated in response to low concentrations of prostaglandin $E_1$, and $E_2$, in the presence of noradrenaline, but treatment with inhibitors of prostaglandin synthesis, indomethacin, aspirin or eicosa-5,8,11,14-tetraynoic acid did not reduce the vasodilator response. Attempts to extract a prostaglandin in the bathing medium were unsuccessful.

6 The involvement of a purine nucleotide appeared unlikely since the ear artery dilated only in response to fairly high concentrations of adenosine 5′-triphosphate (ATP), adenosine 5′-diphosphate (ADP) and adenosine 5′-monophosphate (AMP). Furthermore, dipyridamole, an inhibitor of adenosine uptake, enhanced dilatation due to exogenous ATP but not to periarterial nerve stimulation.

7 It is concluded that the central artery of the rabbit ear has a vasodilator innervation but the identity of the transmitter remains to be established.

## Introduction

The isolated and perfused central artery of the rabbit ear is often used as a model preparation in studies on the physiology and pharmacology of vascular neuroeffector systems (De La Lande & Rand, 1965; De La Lande, Frewin & Waterson, 1967; Bevan & Waterson, 1971; Kalsner, 1972a, b). Electrical stimulation of the periarterial nerves produces constrictions of the artery which are frequency-dependent and reproducible. The marked reduction or abolition of these responses by superior cervical ganglionectomy, reserpine and guanethidine and their potentiation by cocaine point to mediation by sympathetic nerves (De La Lande & Rand, 1965; De La Lande *et al.*, 1967; Kalsner, 1972a). In addition, application of a histochemical fluorescence technique reveals a fluorescent band at the adventitial-medial junction, indicative of catecholamine containing nerve terminals, which is not detectable after sympathectomy or reserpine treatment (Waterson & Smale, 1967; De La Lande, Hodge, Lazner, Jellet & Waterson, 1970).

Evidence is presented in this paper which points to the additional presence of a vasodilator innervation to the central artery of the rabbit ear which is also activated by electrical stimulation of the same parameters used to excite sympathetic constrictor fibers and blocked by treatment with reserpine. The significance of this finding is discussed.

## Methods

The central arteries were taken from both ears of rabbits under urethane anaesthesia (7 ml/kg of a 25% solution, i.p.) and cannulated at both ends essentially as described by De La Lande & Rand (1965) and De La Lande *et al.* (1967). The vessels were suspended in individual 10 or 15 ml muscle baths containing Krebs-Henseleit (Krebs) solution of the following composition (mM): NaCl, 115.3; KCl, 4.6; $CaCl_2$, 2.3; $MgSO_4$, 1.1; $NaHCO_3$, 22.1; $KH_2PO_4$, 1.1; glucose, 7.8; with disodium edetate added (0.03 mM) to retard heavy metal catalyzed oxidation of catecholamines. The Krebs solution was kept at 37°C and continuously aerated with 95% $O_2$ and 5% $CO_2$. A gravity-feed apparatus was used to perfuse the vessels at a constant pressure of 85 cm of water with warmed (37°C) and oxygenated Krebs solution. A record of the rate of flow of Krebs through each artery was provided by an Andrews (1952) outflow recorder writing on a slowly revolving smoked kymograph drum. The height of each vertical stroke of the writing lever is proportional to the volume of air displaced from the outflow recorder over an 8 s period and thus to the volume of Krebs flowing through the vessel lumen and into the recorder during that time interval. Other details and a diagram of the perfusion apparatus are given elsewhere (Kalsner, 1972a).

The procedure used for stimulation of the periarterial nerves was described previously by De La Lande & Rand (1965). A set of platinum electrodes was arranged around the proximal end of each artery over the area where the perfusion cannula lay within the artery. The nerves were stimulated at supramaximal voltage with biphasic pulses of 1 ms pulse width delivered by a Grass model S5 stimulator. Supramaximal voltage was determined for each preparation by stimulating initially at 5 Hz (beginning with 10 V and increasing in 5 V increments) and recording the vasoconstriction produced. A voltage which was about 25% above that which produced maximum vasoconstriction was routinely used in all experiments.

The drugs used were (−)-noradrenaline bitartrate, cocaine, phentolamine, propranolol and methoxamine hydrochlorides, acetylcholine chloride, histamine dihydrochloride, guanethidine sulphate, atropine sulphate, bretylium tosylate, mepyramine maleate, prostaglandins $E_1$, $E_2$ and $F_{2\alpha}$ (Upjohn), adenosine-5′-triphosphate disodium salt (ATP; Nutritional Biochemicals), adenosine-5′-diphosphate monosodium salt (ADP), adenosine-5′-monophosphate (AMP), indomethacin (Merck, Sharp & Dohme), acetylsalicylic acid (aspirin), eicosa-5,8,11,14-tetraynoic acid (Hoffmann-La Roche) and dipyridamole (Boehringer Ingelheim).

All drug solutions were prepared fresh on the day of use and kept chilled in an ice bucket. All dilutions of drugs were in 0.9% w/v NaCl solution with the exception of prostaglandin $E_1$ and $E_2$, eicosa-5,8,11,14-tetraynoic acid and indomethacin which were diluted in minimal volumes of ethanol. The volume of ethanol added to the muscle chambers was routinely kept below 0.03 ml (58 mM) except when obtaining cumulative dose-response curves to the prostaglandins in which case the total volume used was 0.06 ml. These volumes of ethanol alone had no detectable effect on the responses of the rabbit ear artery.

All drug concentrations refer to the final concentrations in the muscle chambers in g/ml. The sympathomimetics and histamine are expressed in terms of the weight of the base and all other compounds either as the pure substance or salt, as indicated above. Reserpine was dissolved in 10% ascorbic acid and administered intramuscularly 18-24 h before death at 0.2, 0.5 or 2.0 mg/kg.

For extraluminal administration the drug under study was added to the muscle chamber containing the Krebs solution bathing the adventitial surface of the artery. For intraluminal administration the drug was dissolved in a reservoir of Krebs solution, at the desired concentration, and the flow of drug-treated or control Krebs solution through the vessel lumen was determined by means of a 2-way stopcock located above the proximal end of the artery.

## Results

### *Effects of periarterial nerve stimulation on arterial tone*

The perfused central artery of the rabbit ear does not show spontaneous activity or basal tone and the effects of periarterial nerve stimulation in the untreated preparation were described previously (Kalsner, 1972a). To investigate the possibility of a vasodilator innervation it was necessary to maintain a background level of arterial tone. This was accomplished by adding extraluminally, to the muscle chamber, a concentration of noradrenaline (5 to 30 ng/ml) sufficient to maintain about a 75% reduction in intraluminal flow. The periarterial nerves were then stimulated continuously for 2 min at either 2 or 5 Hz. Intraluminal flow decreased during stimulation indicative of the release of sympathetic transmitter and increased to

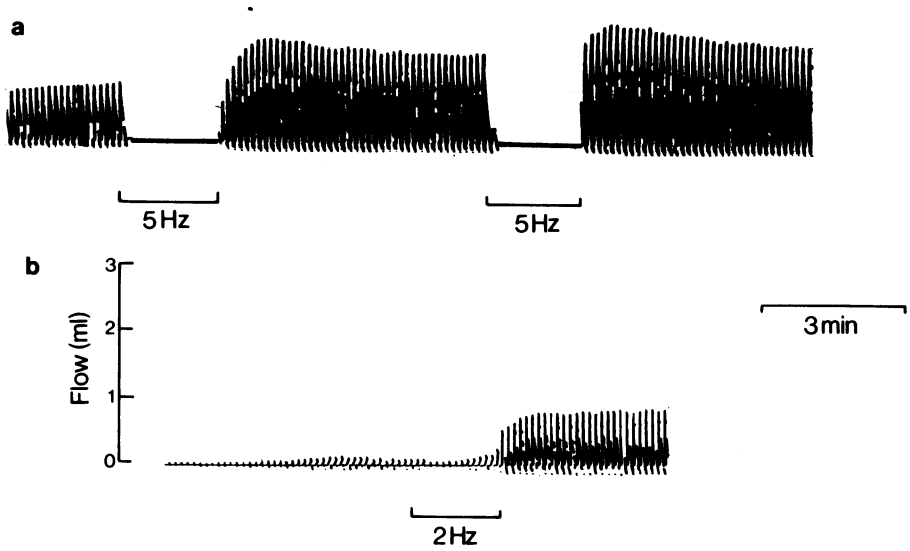


**Fig. 1** Responses of the central artery of the rabbit ear to periarterial nerve stimulation. The duration of stimulation is indicated by enclosed horizontal bars. (a) Vessel exposed to noradrenaline (30 ng/ml) and stimulated twice at 5 Hz. (b) Vessel exposed to noradrenaline (0.2 μg/ml) and stimulated at 2 Hz.

above the pre-stimulation level after the cessation of stimulation. A second period of electrical stimulation without washout of the muscle chamber, usually led to a further increase in flow after the termination of stimulation (Figure 1a). This finding was suggestive of the release of a vasodilator substance whose effects long outlasted those of the constrictor transmitter. The dilator response was not dependent on obtaining a distinct constriction to nerve stimulation since periarterial stimulation in the presence of a concentration of noradrenaline sufficient in itself to virtually obliterate flow in the artery still led to a detectable post-stimulation dilatation (Figure 1b).

*The vasodilator response*

Vasodilatation was usually detected after the cessation of stimulation over the same frequency ranged used to excite vasoconstrictor nerves (0.5 to 5 Hz) and was undiminished in the presence of cocaine (10 μg/ml) or when methoxamine (50 ng to 0.2 μg/ml) or histamine (50 to 100 ng/ml) were added to the muscle chambers in place of noradrenaline to maintain tone. Vasodilatation after a 2 min period of nerve stimulation was also evident when tone was sustained by an intraluminal infusion of noradrenaline (10 to 30 ng/ml) rather than by its addition extraluminally to the muscle chamber. In a few preparations in which the constrictor response to nerve stimulation at low frequencies was small or absent it was noted that periarterial nerve stimulation, in the presence of noradrenaline, still led to a clearly detectable vasodilatation.

To obtain more direct evidence for a vasodilator innervation to the central ear artery the vessels were exposed to guanethidine (10 or 20 μg/ml) for 15 or 30 min to deplete catecholamine stores. After an additional interval (usually 15 min) with frequent changes of the bath fluid, a background level of tone was produced with noradrenaline (3 to 30 ng/ml). Stimulation of the periarterial nerves for about 3 min at 2 or 5 Hz gave a pure dilatation now unmasked by blockade of the vasoconstrictor component of periarterial nerve activation (Figure 2a). This was observed in 35 preparations. The dilatation was not reversed abruptly when stimulation was stopped but decreased only gradually with time. In some preparations the vessel remained dilated above the background level for up to 15 min until the muscle chamber was flushed with Krebs. In other experiments vessels were exposed to bretylium (3 to 10 μg/ml) for 15 min and without washout of the muscle chamber the periarterial nerves stimulated at 2 or 5 Hz. The results obtained in six preparations were similar to those with guanethidine and a typical record is presented in Figure 2c.

Additional evidence for the release of a

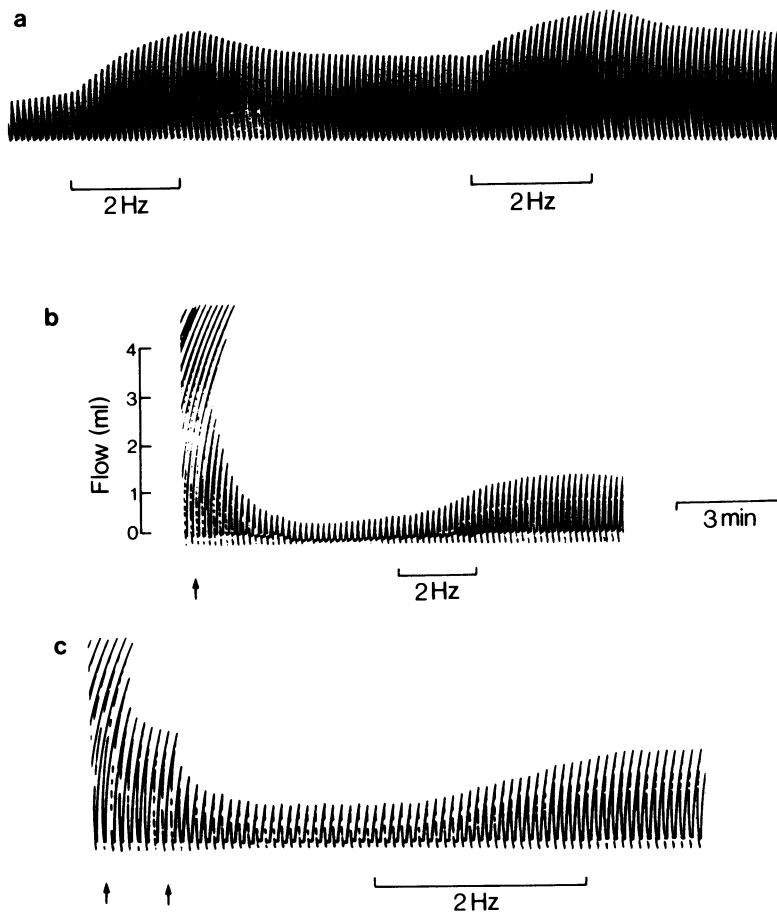


**Fig. 2** Vasodilator response of the central artery of the rabbit ear to periarterial nerve stimulation. (a) Vessel pretreated with guanethidine (10 μg/ml) for 30 min and 15 min later exposed to noradrenaline (20 ng/ml) and stimulated twice at 2 Hz. (b) Vessel pretreated with phentolamine (3 μg/ml) and propranolol (1 μg/ml) for 15 min and, without washout, exposed to histamine (0.1 μg/ml; arrow) and stimulated at 2 Hz. (c) Vessel pretreated with bretylium (3 μg/ml) for 15 min and, without washout, exposed cumulatively to noradrenaline (1 and 3 ng/ml; arrows) and stimulated at 2 Hz.

vasodilator material upon stimulation of the periarterial nerves was obtained in experiments in which both the α- and β-adrenoceptors were blocked by phentolamine (3 or 5 μg/ml) and propranolol (1 μg/ml) and histamine was used to maintain background tone. Stimulation at 2 or 5 Hz led to a distinct dilator response in four out of six vessels tested (Figure 2b).

*Effects of drugs on the vasodilator response*

Arteries of rabbits pretreated with a moderate dose of reserpine (2 mg/kg for 18 h) did not respond to periarterial nerve stimulation with constriction or dilatation in a total of four preparations examined. However, it was observed in four out of five preparations pretreated with reserpine at 0.2-0.5 mg/kg that although the constrictor response was absent periarterial stimulation at 2 or 5 Hz in the presence of noradrenaline gave a clear dilator response.

The dilator response to periarterial nerve stimulation in untreated preparations constricted with noradrenaline, or in those treated with guanethidine, was not reduced in the presence extraluminally of atropine (0.1 to 10 μg/ml), mepyramine (0.3 μg/ml) or propranolol (1 μg/ml); nor did physostigmine (1 or 3 μg/ml), an inhibitor

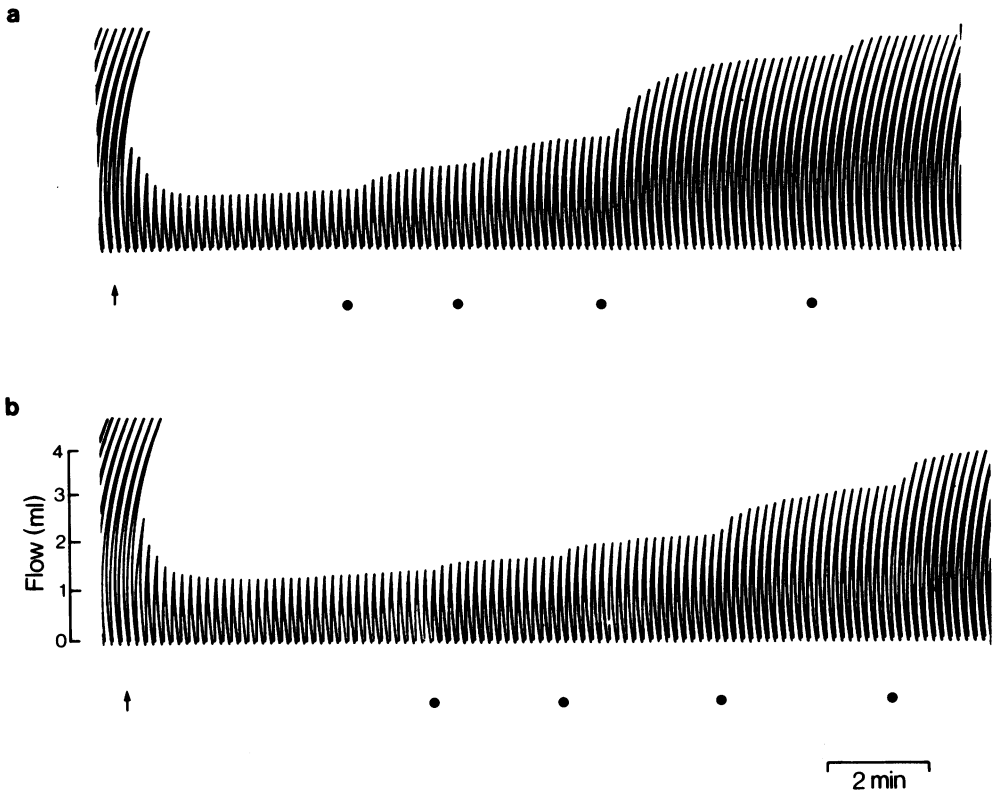


**Fig. 3** Responses of the central artery of the rabbit ear to prostaglandin $E_2$ and ATP. (a) Vessel constricted with noradrenaline (30 ng/ml; arrow) and exposed cumulatively to prostaglandin $E_2$ (10 and 30 ng, 0.1 and 0.3 μg/ml; dots). (b) Vessel constricted with noradrenaline (30 ng/ml) and exposed to ATP (0.3, 1, 3 and 10 μg/ml; dots).

of cholinesterase, enhance the dilatation. Atropine (10 μg/ml) and physostigmine (3 or 10 μg/ml) had no discernible effect on the magnitude of the dilatation even when administered simultaneously by both the extra and intraluminal routes, beginning 15 to 30 min prior to nerve stimulation. In contrast, dilator responses to acetylcholine administered by intraluminal perfusion (10 ng to 0.2 μg) were enhanced routinely by physostigmine and blocked by atropine. Neither histamine (0.3 ng to 0.5 μg/ml) nor acetylcholine (1 ng to 1 μg/ml) dilated the partially constricted central ear artery when added extraluminally to the muscle chambers, providing additional evidence for the lack of involvement of these substances.

The central artery of the rabbit ear responded to low concentrations of prostaglandin $E_1$ (30 to 100 ng/ml) and $E_2$ (10 to 30 ng/ml) with dilatation when these agents were administered extraluminally in the presence of a background level of noradrenaline tone. A typical kymograph record of one such experiment is presented in Figure 3a. In contrast, the vessel constricted in response to prostaglandin $F_{2\alpha}$ (0.3 to 1 μg/ml). The possibility that the vasodilator response to periarterial nerve stimulation is mediated by a prostaglandin was considered. However, inhibitors of prostaglandin synthesis, aspirin (10 to 100 μg/ml), indomethacin (1 to 30 μg/ml) or eicosa-5,8,11,14-tetraynoic acid (3 to 10 μg/ml), did not reduce the magnitude of the dilator response in eight, four and five preparations, respectively. The vasodilatation following the cessation of nerve stimulation in untreated vessels partially constricted with noradrenaline, or the unmasked vasodilator response in vessels pre-treated with guanethidine or bretylium did not differ materially when compared before and after treatment with inhibitors of prostaglandin synthesis.

In addition, attempts to extract a prostaglandin from the fluid bathing the central ear artery after a

prolonged period of nerve stimulation were unsuccessful. Vessels were stimulated at 5 Hz for periods of up to 60 min and the extraluminal bath fluid extracted with ethylacetate and re-dissolved after evaporation in a small volume of Krebs (0.5-1.0 ml), as described previously by others (Tothill, Rathbone & Willman, 1971; Hedqvist & Euler, 1972). The addition of these volumes to segments of rat ileum or strips of guinea-pig stomach did not elicit contractions although these preparations responded to 0.3 ng/ml of prostaglandin $E_2$ and $E_1$ with contractions.

The central ear artery was constricted with noradrenaline and exposed to ATP, ADP or AMP over a broad concentration range to determine whether these substances could be considered as candidates for mediation of the vasodilator response. This seemed unlikely since these compounds produced detectable dilatation only when added to the muscle chambers in the high concentrations of 0.3 to 10 $\mu$g/ml (e.g. Figure 3b). The possibility that the dilator response to nerve stimulation was mediated by an adenosine compound was explored further using dipyridamole, an inhibitor of adenosine inactivation (Kolassa, Pfleger & Rummel, 1970; Burnstock, 1972; Hopkins, 1973). Concentrations of 1 to 3 $\mu$g/ml of dipyridamole did not increase the magnitude of the dilator response in 14 preparations examined, although responses to added ATP were distinctly enhanced.

## Discussion

Evidence has been presented that the central artery of the rabbit ear is supplied with a vasodilator innervation as well as a sympathetic constrictor system. The usual response of the isolated and perfused artery to periarterial nerve stimulation is constriction. However, if a background level of tone is maintained by the addition of noradrenaline or some other agonist to the muscle chamber it is observed that following the cessation of stimulation, flow through the vessel increases to above the pre-stimulation level. A pure dilator response to periarterial nerve stimulation was unmasked by blockade of adrenergic nerves with guanethidine or bretylium or by blockade of the vasoconstrictor effects of noradrenaline with $\alpha$- and $\beta$-receptor blocking agents.

Other reports have appeared pointing to the presence of a non-adrenergic, non-cholinergic dilator innervation to the cutaneous vascular beds of various species. For example, stimulation of the sympathetic outflow to the hindlimbs of dogs or cats, after blockade of adrenergic neurons, produces atropine-resistant vasodilatation (Beck, Pollard, Kayaalp & Weiner, 1966; Brody, 1966; Zimmerman, 1966, 1968). Beck *et al.* (1966) have shown that there is an initial, rapidly developing, dilatation in the hindlimbs of dogs which is blocked by atropine and a sustained component of dilatation of slow onset and termination which is not blocked by any of the classical groups of antagonists. Zimmerman (1966, 1968) has demonstrated a prolonged dilatation restricted to the cutaneous vascular bed of the dog hindlimb, and to a lesser extent of the cat, apparent after adrenergic neurone blockade and mediated by an unidentified transmitter. He also identified a rapidly developing dilatation blocked by atropine and an additional component limited to the muscle vessels which could be blocked by an antihistamine.

Ballard, Aboud & Mayer (1970) also observed a transient vasodilatation in the isolated and perfused gracilis muscle of the dog which was blocked by atropine but the sustained dilator response obtained in the paw was not blocked by a combination of an antihistamine and atropine.

In the present experiments it was observed that treatment with reserpine at 2.0 mg/kg, to deplete the sympathetic nerves of catecholamines, eliminated both constrictor and dilator responses to periarterial nerve stimulation whereas lower doses (0.2-0.5 mg/kg) selectively eliminated the constriction. This finding may bear on the conflicting reports of other investigators on the effect of reserpine on the vasodilatation observed in the cutaneous vascular bed of the dog hindlimb (Beck & Brody, 1961; Ballard *et al.*, 1970; Pollard & Beck, 1971).

Reports of a vasodilator innervation to vascular tissue in the rabbit have also appeared. Hughes & Vane (1967, 1970) observed a relaxation of spontaneously active strips of rabbit portal vein which was not blocked by atropine, in response to electrical stimulation after adrenergic blockade. The mediator of this response has not yet been identified.

Attempts were made to identify the mediator of the vasodilator response in the isolated and perfused segment of the central artery of the rabbit ear. The possibility that a prostaglandin is released in response to electrical stimulation was considered, since it has been proposed that these lipids may be modulators of adrenergic neurotransmission in blood vessels (Hedqvist, 1972; Kadowitz, Sweet & Brody, 1972). Also, Beck *et al.* (1966) have suggested that prostaglandins mediate the prolonged vasodilatation observed in the dog hindlimb. Attempts to inhibit the vasodilator response to nerve stimulation by treatment with inhibitors of

prostaglandin synthesis were unsuccessful. Prostaglandin released by nerve stimulation is believed to represent newly synthesized material but aspirin, indomethacin or the potent and irreversible inhibitor eicosa-5,8,11,14-tetraynoic acid (Downing, Ahern & Bachta, 1970; Ferreira, Moncada & Vane, 1971; Hedqvist, Stjarne & Wennmalm, 1971; Samuelsson & Wennmalm, 1971) had no detectable effect on the magnitude of the dilatation.

Burnstock (1972) has recently summarized evidence for the existence of purinergic nerves particularly to the gastrointestinal tract and bladder. The possibility that the vasodilator response observed in the present experiments is related to the release of ATP or other purine nucleotide was considered. ATP, ADP and AMP were all only weakly active in dilating the partially constricted ear artery. In addition, dipyridamole, an inhibitor of adenosine uptake which potentiates the responses to purinergic nerve stimulation had no discernible effect although responses to added ATP were enhanced. An additional point against the likelihood that a purine nucleotide is involved in the vasodilator response was the finding that a moderate concentration of reserpine eliminated the vasodilatation. Reserpine does not appear to deplete the large opaque vesicles believed to be representative of purinergic nerves (Burnstock, 1972).

The responses of the vascular bed of the rabbit ear to changes in temperature has provided suggestive evidence of a vasodilator system (Grant & Bland, 1932). Holton & Perry (1951) demonstrated a dilatation of the vessels of the rabbit ear in response to stimulation of the peripheral end of the cut ventral auricular nerve and attributed this to antidromic vasodilatation as had Feldberg (1926) previously. However, Feldberg (1926) and Grant & Bland (1932) have shown that each of the four sensory nerves to the ear contains efferent vasomotor fibers as well. Holton (1959) subsequently identified ATP in the effluent following auricular nerve and to some extent sympathetic nerve activation, but concluded that in the case of the auricular nerve it was probably released in association with an unidentified transmitter substance.

Although antidromic vasodilatation cannot be excluded as a factor in the present experiments it appears unlikely in light of the observed antagonism by reserpine and the report by Holton & Perry (1951) that dilatation due to sensory nerve activation is depressed by physostigmine. No such effect of the cholinesterase inhibitor was observed in the present experiments.

Holton & Rand (1962), using a photocell method, observed an initial constriction followed by a dilatation of the vascular bed of the intact rabbit ear in response to sympathetic nerve stimulation. In a thorough study which included adrenergic neurone blockade, they found that the dilatation was antagonized by atropine and enhanced by physostigmine. The data presented here cannot be explained by cholinergic mediation, however, since neither atropine nor physostigmine, even in high concentrations, influenced the magnitude of the dilator response, although responses to administered acetylcholine were blocked and enhanced, respectively, by these agents. In addition, the dilator response was eliminated by pretreatment of rabbits with reserpine. In this context, it should be noted that Holton & Rand (1962) measured flow primarily in the small vessels of the distal third of the ear whereas in the present experiments the proximal portion of the central ear artery was utilized. Further, the dilator effect which these workers observed did not appear to involve the resistance vessels to any significant extent.

The present findings of a dilator response to periarterial stimulation in a short segment of an isolated and perfused artery suggests the possibility of a distinct dilator innervation to the vascular smooth muscle cells. The release of a transmitter from such a preparation may provide excellent opportunities for its future isolation and characterization.

The results obtained here also indicate the need to reconsider carefully the previous studies on periarterial nerve stimulation of the ear vessels. Such data may need to be re-interpreted due to the apparent simultaneous release of both vasodilator and vasoconstrictor substances. The possibility that other vascular beds might also possess a vasodilator innervation remains to be explored.

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